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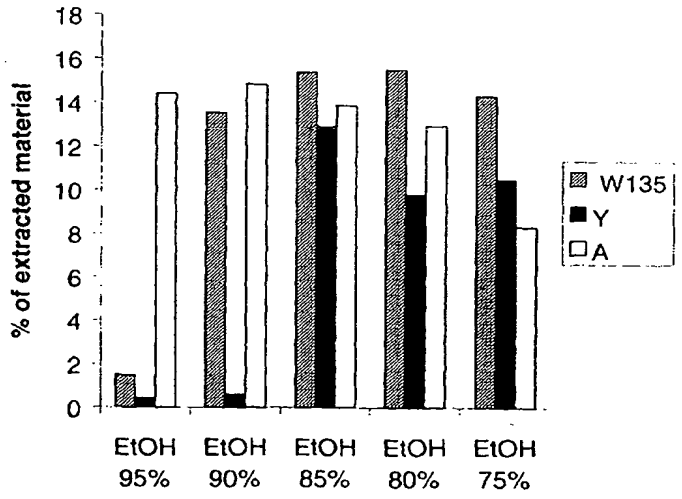
(54) Title: CAPSULAR POLYSACCHARIDE SOLUBILISATION AND COMBINATION VACCINES



(57) Abstract: Precipitated bacterial capsular polysaccharides can be efficiently re-solubilised using alcohols as solvents. The invention provides a process for purifying a bacterial capsular polysaccharide, comprising the steps of (a) precipitation of said polysaccharide, followed by (b) solubilisation of the precipitated polysaccharide using ethanol. CTAB can be used for step (a). The material obtained, preferably following hydrolysis and sizing, can be conjugated to a carrier protein and formulated as a vaccine. Also, in vaccines comprising saccharides from the serogroups A and C, the invention provides that the ratio (w/w) of MenA saccharide : MenC saccharide is >1.

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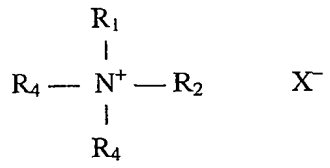
Precipitation and ethanol solubilisation

Many techniques for precipitating soluble polysaccharides are known in the art. Preferred methods use one or more cationic detergents. The detergents preferably have the following general formula:



wherein: R_1, R_2 and R_3 are the same or different and each signifies alkyl or aryl; or R_1 and R_2 together with the nitrogen atom to which these are attached form a 5- or 6-membered saturated heterocyclic ring, and R_3 signifies alkyl or aryl; or R_1, R_2 and R_3 together with the nitrogen atom to which these are attached form a 5- or 6-membered heterocyclic ring, unsaturated at the nitrogen atom,

R_4 signifies alkyl or aryl, and

X^- signifies an anion.

Particularly preferred detergents for use in the method are tetrabutylammonium and cetyltrimethylammonium salts (*e.g.* the bromide salts). Cetyltrimethylammonium bromide ('CTAB') is particularly preferred [8]. CTAB is also known as hexadecyltrimethylammonium bromide, cetrimonium bromide, Cetavlon and Centimide. Other detergents include hexadimethrine bromide and myristyltrimethylammonium salts.

Capsular polysaccharides are released into media during culture. Accordingly, the starting material for precipitation will typically be the supernatant from a centrifuged bacterial culture or will be a concentrated culture.

The precipitation step may be selective for polysaccharides, but it will typically also co-precipitate other components (*e.g.* proteins, nucleic acid *etc.*).

Precipitated polysaccharide may be collected by centrifugation prior to solubilisation.

After precipitation, the polysaccharide (typically in the form of a complex with the cationic detergent) is re-solubilised. It is preferred to use a solvent which is relatively selective for the polysaccharide in order to minimise contaminants (*e.g.* proteins, nucleic acid *etc.*). Ethanol has been found to be advantageous in this respect, and it is highly selective for the CTAB-polysaccharide complex. Other lower alcohols may be used (*e.g.* methanol, propan-1-ol, propan-2-ol, butan-1-ol, butan-2-ol, 2-methyl-propan-1-ol, 2-methyl-propan-2-ol, diols *etc.*)

The ethanol is preferably added to the precipitated polysaccharide to give a final ethanol concentration (based on total content of ethanol and water) of between 50% and 95% (*e.g.* around 55%, 60%, 65%, 70%, 75%, 80%, 85%, or around 90%), and preferably between 75% and 95%. The

optimum final ethanol concentration may depend on the serogroup of the bacterium from which the polysaccharide is obtained.

The ethanol may be added to the precipitated polysaccharide in pure form or may be added in a form diluted with a miscible solvent (*e.g.* water). Preferred solvent mixtures are ethanol:water mixtures, with a preferred ratio of between around 70:30 and around 95:5 (*e.g.* 75:25, 80:20, 85:15, 90:10).

Compared with conventional processes for preparing capsular polysaccharides, the two-step process of precipitation followed by ethanol extraction is quicker and simpler.

In contrast to the process described in ref. 9, the process uses cationic detergent rather than anionic detergent. Unlike the process of ref. 10, the polysaccharide is re-solubilised using ethanol, rather than by cation exchange using calcium or magnesium salts. Unlike the process of ref. 11, precipitation does not require an inert porous support. Furthermore, unlike prior art processes, alcohol is used to re-solubilise the polysaccharide rather than to precipitate it.

The bacterial capsular polysaccharide will usually be from *Neisseria.* Preferably it is from *N.meningitidis*, including serogroups A, B, C, W135 & Y. Preferred serogroups are A, W135 & Y.

The process is also suitable for preparing capsular polysaccharide from *Haemophilus influenzae* (particularly type B, or 'Hib') and from *Streptococcus pneumoniae* (pneumococcus).

Further processing of the solubilised polysaccharide

After re-solubilisation, the polysaccharide may be further treated to remove contaminants. This is particularly important in situations where even minor contamination is not acceptable (*e.g.* for human vaccine production). This will typically involve one or more steps of filtration.

Depth filtration may be used. This is particularly useful for clarification.

Filtration through activated carbon may be used. This is useful for removing pigments and trace organic compounds. It can be repeated until, for example, $OD_{275nm}<0.2$.

Size filtration or ultrafiltration may be used.

Once filtered to remove contaminants, the polysaccharide may be precipitated for further treatment and/or processing. This can be conveniently achieved by exchanging cations (*e.g.* by the addition of calcium or sodium salts).

The polysaccharide may be chemically modified. For instance, it may be modified to replace one or more hydroxyl groups with blocking groups. This is particularly useful for MenA [12]. Polysaccharides from serogroup B may be N-propionylated [13].

The (optionally modified) polysaccharide will typically be hydrolysed to form oligosaccharides. This is preferably performed to give a final average degree of polymerisation (DP) in the oligosaccharide of less than 30 (*e.g.* between 10 and 20, preferably around 10 for serogroup A; between 15 and 25 for serogroups W135 and Y, preferably around 15-20; *etc.*). Oligosaccharides are preferred to polysaccharides for use in vaccines. DP can conveniently be measured by ion exchange chromatography or by colorimetric assays [14].

If hydrolysis is performed, the hydrolysate will generally be sized in order to remove short-length oligosaccharides. This can be achieved in various ways, such as ultrafiltration followed by ion-exchange chromatography. Oligosaccharides with a degree of polymerisation of less than or equal to about 6 are preferably removed for serogroup A, and those less than around 4 are preferably removed for serogroups W135 and Y.

To enhance immunogenicity, polysaccharides or oligosaccharides of the invention are preferably conjugated to a carrier (Figure 18). Conjugation to carrier proteins is particularly useful for paediatric vaccines [*e.g.* ref. 15] and is a well known technique [*e.g.* reviewed in refs. 16 to 24, *etc.*].

Preferred carrier proteins are bacterial toxins or toxoids, such as diphtheria or tetanus toxoids. The CRM_{197} diphtheria toxoid [25, 26, 27] is particularly preferred. Other suitable carrier proteins include the *N.meningitidis* outer membrane protein [28], synthetic peptides [29, 30], heat shock proteins [31, 32], pertussis proteins [33, 34], cytokines [35], lymphokines [35], hormones [35], growth factors [35], artificial proteins comprising multiple human $CD4^+$ T cell epitopes from various pathogen-derived antigens [36, protein D from *H.influenzae* [37], toxin A or B from *C.difficile* [38], *etc.* It is possible to use mixtures of carrier proteins.

Conjugates with a saccharide:protein ratio (w/w) of between 0.5:1 (*i.e.* excess protein) and 5:1 (*i.e.* excess saccharide) are preferred, and those with a ratio between 1:1.25 and 1:2.5 are more preferred.

A single carrier protein may carry multiple different saccharides [39]. Conjugates may be used in conjunction with free carrier protein [40].

Any suitable conjugation reaction can be used, with any suitable linker where necessary.

The saccharide will typically be activated or functionalised prior to conjugation. Activation may involve, for example, cyanylating reagents such as CDAP (*e.g.* 1-cyano-4-dimethylamino pyridinium tetrafluoroborate [41, 42, *etc.*]). Other suitable techniques use carbodiimides, hydrazides, active esters, norborane, p-nitrobenzoic acid, N-hydroxysuccinimide, S-NHS, EDC, TSTU; see also the introduction to reference 22).

Linkages via a linker group may be made using any known procedure, for example, the procedures described in references 43 and 44. One type of linkage involves reductive amination of the